

CLAIMS

What is claimed is:

1. A method of quantitatively determining the stability of a test protein under native conditions, the method comprising:
  - 5 (a) providing a test protein;
  - (b) contacting the protein with an exchange buffer comprising a denaturant and deuterium, the exchange buffer having a denaturant concentration;
  - (c) contacting the test protein with a mass spectrometry matrix medium;
  - 10 (d) determining a change in mass of the test protein by mass spectrometry;
  - (e) varying the denaturant concentration of the exchange buffer;
  - (f) repeating steps (a)-(e) a desired number of times; and
  - 15 (g) quantitatively determining protein stability based on the change in mass of the test protein as a function of denaturant concentration, whereby the stability of a test protein under native conditions is quantitatively determined.
- 20 2. The method of claim 1, wherein the test protein is disposed in a crude cell lysate.
3. The method of claim 1, wherein the test protein is associated with a disease phenotype.
- 25 4. The method of claim 3, wherein the disease phenotype is characterized by protein misfolding.
5. The method of claim 1, wherein the test protein has a mass of less than 1,000,000 daltons.
- 30

6. The method of claim 1, wherein the test protein is a multimeric protein.

7. The method of claim 1, wherein the test protein is disposed on  
5 a microtiter plate.

8. The method of claim 7, wherein a plurality of test proteins are disposed on the microtiter plate.

10 9. The method of claim 8, wherein the method further comprises the step of repeating steps (a)-(g) for each test protein disposed on the microtiter plate.

10. The method of claim 1, wherein the test protein is provided in  
15 picomolar or greater amounts.

11. The method of claim 1, wherein the test protein is *in vivo*.

12. The method of claim 1, wherein the denaturant is a chemical  
20 denaturant.

13. The method of claim 12, wherein the chemical denaturant is selected from the group consisting of detergents, guanidinium chloride and urea.  
25

14. The method of claim 1, wherein the mass spectrometry matrix ~~material~~ <sup>medium</sup> is a MALDI mass spectrometry matrix ~~material~~ <sup>medium</sup> and the mass spectrometry is MALDI mass spectrometry.

15. The method of claim 14, wherein the MALDI mass spectrometry matrix material is selected from the group consisting of  
30

sinapinic acid,  $\alpha$ -cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, 2,5-dihydroxyacetophenone and 3-amino-4-hydroxybenzoic acid.

16. The method of claim 1, wherein the step of quantitatively  
5 determining protein stability further comprises fitting data comprising a change in mass of the test protein as a function of denaturant concentration to the equation  $\Delta\text{Mass} = \Delta M_{\infty} + (\Delta M_0 - \Delta M_{\infty})e^{-(\Delta G_f + m[\text{Denat.}]/RT)/(1+K_{\text{fold}})}$ , wherein  $K_{\text{fold}} = e^{-(\Delta G_f + m[\text{Denat.}]/RT)}$  and  $\Delta G_f$  is the stability of the test protein under native conditions.

10

17. The method of claim 16, wherein the step of quantitatively determining protein stability is performed using a computer program.

18. The method of claim 1, wherein the step of quantitatively  
15 determining protein stability comprises determining a free energy of folding from a denaturation curve based on the change of mass of the protein, the free energy of folding being indicative of the stability of a test protein.

19. The method of claim 1, wherein the step of quantitatively  
20 determining a change in mass of the test protein is based on the exchange of deuterium for hydrogen, the exchange being dependent on slow-exchanging hydrogens in the test protein.

20. The method of claim 1, further comprising providing a  
25 reference protein with the test protein.

21. The method of claim 1, further comprising:  
(a) contacting the test protein with a reverse phase chromatography matrix; and  
30 (b) eluting the test protein from the chromatography matrix.

22. The method of claim 21, further comprising contacting the test protein with an acid before contacting the test protein with the reverse phase chromatography matrix.

- 5           23. A method of detecting a binding event involving a test protein with a test ligand, the method comprising:
- (a) providing a test protein;
  - (b) providing a test ligand;
  - (c) contacting the test ligand with the test protein to form a test  
10           mixture;
  - (d) contacting the test mixture with an exchange buffer comprising a denaturant and deuterium, the exchange buffer having a denaturant concentration;
  - (e) contacting the test mixture with a mass spectrometry matrix  
15           medium;
  - (f) determining a change in mass of the test protein by mass spectrometry;
  - (g) varying the denaturant concentration of the exchange buffer;
  - (h) repeating steps (a)-(g) a desired number of times; and
  - 20           (i) analyzing the change in mass of the test protein as a function of denaturant concentration, whereby a binding event involving the test protein and the test ligand is detected.

24. The method of claim 23, wherein the test protein is disposed in  
25           a crude cell lysate.

25. The method of claim 23, wherein the test protein is associated with a disease phenotype.

30           26. The method of claim 23, wherein the disease phenotype is characterized by protein misfolding.

27. The method of claim 23, wherein the test protein has a mass of less than 1,000,000 daltons.

28. The method of claim 23, wherein the test protein is a  
5 multimeric protein.

29. The method of claim 23, wherein the test protein is disposed on a microtiter plate.

10 30. The method of claim 29, wherein a plurality of test proteins are disposed on the microtiter plate.

31. The method of claim 30, wherein the method further comprises the step of repeating steps (a)-(i) for each test protein disposed on the  
15 microtiter plate.

32. The method of claim 23, wherein the test protein is provided in picomolar or greater amounts.

20 33. The method of claim 23, wherein the test protein is *in vivo*.

34. The method of claim 23, wherein the denaturant is a chemical denaturant.

25 35. The method of claim 34, wherein the denaturant is selected from the group consisting of detergents, guanidinium chloride and urea.

36. The method of claim 23, wherein the mass spectrometry matrix material is a MALDI mass spectrometry matrix material and the mass  
30 spectrometry is MALDI mass spectrometry.

37. The method of claim 36, wherein the MALDI mass spectrometry matrix material is selected from the group consisting of sinapinic acid,  $\alpha$ -cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, 2,5-dihydroxyacetophenone and 3-amino-4-hydroxybenzoic acid.

5

38. The method of claim 23, wherein the analyzing comprises:

- (a) plotting the change in mass of the test protein in the presence of the test ligand as a function of denaturant concentration to generate a first denaturation curve;
- 10 (b) plotting the change in mass of the test protein in the absence of the test ligand as a function of denaturant concentration to generate a second denaturation curve; and
- (c) identifying a change in the position of the first denaturation curve relative to the position of the second denaturation curve, wherein a difference in the positions of the first and second denaturation curves is indicative of a binding event involving the test ligand and the test protein.

15

39. The method of claim 23, wherein the analyzing is performed using a computer program.

20

40. The method of claim 23, further comprising providing a reference protein with the test protein.

25

41. A method of quantitatively determining a change in the stability of a test protein imparted by the association of a test ligand with the test protein, the method comprising:

30

- (a) providing a test protein;
- (b) providing a test ligand;
- (c) contacting the test ligand with the test protein to form a test mixture;

- 5
- (d) contacting the test mixture with an exchange buffer comprising a denaturant and deuterium, the exchange buffer having a denaturant concentration;
  - (e) contacting the test mixture with a mass spectrometry matrix medium;
  - (f) determining a change in mass of the test protein by mass spectrometry;
  - (g) varying the denaturant concentration of the exchange buffer;
  - (h) repeating steps (a)-(g) a desired number of times;
  - 10 (i) calculating the stability of the test protein in the presence of the test ligand; and
  - (j) quantitatively comparing the stability of the test protein in the presence of the test ligand with the stability of the protein in the absence of ligand to thereby generate a change in the stability of a test protein imparted by the association of a test ligand with the test protein.
- 15

20 42. The method of claim 41, wherein the test protein is disposed in a crude cell lysate.

43. The method of claim 41, wherein the test protein is associated with a disease phenotype.

25 44. The method of claim 41, wherein the disease phenotype is characterized by protein misfolding.

45. The method of claim 41, wherein the test protein has a mass of less than 1,000,000 daltons.

30 46. The method of claim 41, wherein the test protein is a multimeric protein.

47. The method of claim 41, wherein the test protein is disposed on a microtiter plate.

48. The method of claim 47, wherein a plurality of test proteins are disposed on the microtiter plate.

49. The method of claim 48, wherein the method further comprises the step of repeating steps (a)-(i) for each test protein disposed on the microtiter plate.

10

50. The method of claim 41, wherein the test protein is provided in picomolar or greater amounts.

15

51. The method of claim 41, wherein the test protein is *in vivo*.

52. The method of claim 41, wherein the denaturant is a chemical denaturant.

20

53. The method of claim 52, wherein the denaturant is selected from the group consisting of detergents, guanidinium chloride and urea.

25

54. The method of claim 41, wherein the mass spectrometry matrix material is a MALDI mass spectrometry matrix material and the mass spectrometry is MALDI mass spectrometry.

30

55. The method of claim 54, wherein the MALDI mass spectrometry matrix material is selected from the group consisting of sinapinic acid,  $\alpha$ -cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, 2,5-dihydroxyacetophenone and 3-amino-4-hydroxybenzoic acid.

56. The method of claim 41, wherein the step of calculating the stability of the test protein in the presence of the test ligand further

comprises fitting data comprising a change in mass of the test protein as a function of denaturant concentration to the equation  $\Delta\text{Mass} = \Delta M_{\infty} + (\Delta M_0 - \Delta M_{\infty})e^{-(\langle k_{int} \rangle / (1 + K_{fold}))t}$ , wherein  $K_{fold} = e^{-(\Delta G_f + m[\text{Denat.}]/RT)}$  and  $\Delta G_f$  is the stability of the test protein under native conditions.

5

57. The method of claim 41, wherein the calculating is performed using a computer program.

58. The method of claim 41, wherein the step of calculating the  
10 stability of the test protein in the presence of the test ligand comprises determining a free energy of folding from a denaturation curve based on the change of mass of the protein, the free energy of folding being indicative of the stability of a test protein.

15 59. The method of claim 41, wherein the step of calculating the stability of the test protein in the presence of the test ligand is based on the exchange of deuterium for hydrogen, the amount of exchange being dependent on slow-exchanging hydrogens in the test protein.

20 60. The method of claim 41, further comprising providing a reference protein with the test protein.

61. A method of detecting an improperly folded mutant protein, the method comprising:

- 25 (a) contacting a mutant test protein with an exchange buffer comprising a denaturant and deuterium, the exchange buffer having a denaturant concentration ;
- (b) contacting a mutant test protein with a mass spectrometry matrix medium;
- 30 (c) determining a change in mass of the mutant test protein by mass spectrometry;
- (d) varying the denaturant concentration of the exchange buffer;

- (e) repeating steps (a)-(e) a desired number of times;
- (f) quantitatively determining protein stability based on the change in mass of the mutant test protein as a function of denaturant concentration; and
- 5 (g) comparing the stability of the mutant test protein with the stability of a control, non-mutated test protein, a difference in the stabilities being indicative of an improperly folded mutant protein.

10 62. The method of claim 61, further comprising providing a test protein having an amino acid sequence.

63. The method of claim 62, further comprising introducing a mutation into the amino acid sequence of the test protein to form a mutant  
15 test protein.

64. The method of claim 61, wherein the mutation is introduced at a known position in the amino acid sequence of the test protein.

20 65. The method of claim 61, wherein the mutation is introduced at an unknown position in the amino acid sequence of the test protein.

66. The method of claim 63, wherein a plurality of mutations are introduced into the amino acid sequence of the test protein.  
25

67. The method of claim 61, wherein the mutant test protein is disposed in a crude cell lysate.

68. The method of claim 61, wherein the mutant test protein is  
30 associated with a disease phenotype.

69. The method of claim 61, wherein the disease phenotype is characterized by protein misfolding.

70. The method of claim 61, wherein the mutant test protein has a  
5 mass of less than 1,000,000 daltons.

71. The method of claim 61, wherein the mutant test protein is a multimeric protein.

72. The method of claim 61, wherein the mutant test protein is  
10 disposed on a microtiter plate.

73. The method of claim 72, wherein a plurality of mutant test  
15 proteins are disposed on the microtiter plate.

74. The method of claim 73, wherein the method further comprises  
the step of repeating steps (a)-(g) for each mutant test protein disposed on  
the microtiter plate.

75. The method of claim 61, wherein the test protein is provided in  
20 picomolar or greater amounts.

76. The method of claim 61, wherein the mutant test protein is *in*  
25 *vivo*.

77. The method of claim 61, wherein the denaturant is a chemical  
denaturant.

78. The method of claim 77, wherein the denaturant is selected  
30 from the group consisting of detergents, guanidinium chloride and urea.

79. The method of claim 61, wherein the mass spectrometry matrix material is a MALDI mass spectrometry matrix material and the mass spectrometry is MALDI mass spectrometry.

5           80. The method of claim 79, wherein the MALDI mass spectrometry matrix material is selected from the group consisting of sinapinic acid,  $\alpha$ -cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, 2,5-dihydroxyacetophenone and 3-amino-4-hydroxybenzoic acid.

10           81. The method of claim 61, wherein the step of quantitatively determining protein stability based on the change in mass of the mutant test protein as a function of denaturant concentration comprises fitting data comprising a change in mass of the mutant test protein as a function of denaturant to the equation  $\Delta\text{Mass} = \Delta M_{\infty} + (\Delta M_0 - \Delta M_{\infty})e^{-(k_{\text{int}}/(1+K_{\text{fold}}))t}$ ,  
15 wherein  $K_{\text{fold}} = e^{-(\Delta G_f + m[\text{Denat.}]/RT)}$  and  $\Delta G_f$  is the stability of the mutant test protein.

            82. The method of claim 61, wherein the determining is performed using a computer program.

20           83. The method of claim 61, wherein the step of quantitatively determining protein stability based on the change in mass of the mutant test protein as a function of denaturant concentration comprises determining a free energy of folding from a denaturation curve based on the change of  
25 mass of the protein, the free energy of folding being indicative of the stability of a test protein.

            84. The method of claim 61, wherein the step of quantitatively determining protein stability based on the change in mass of the mutant test  
30 protein as a function of denaturant concentration comprises determining a change in mass of the test protein based on the exchange of deuterium for

hydrogen, the exchange being dependent on slow-exchanging hydrogens in the test protein.

5        85. The method of claim 61, further comprising providing a reference protein with the mutant test protein.

86. The method of claim 61, further comprising:  
      (a) contacting the test protein with a reverse phase chromatography matrix; and  
10        (b) eluting the test protein from the chromatography matrix.

87. The method of claim 86, further comprising contacting the test protein with an acid before contacting the test protein with the reverse phase chromatography matrix.

15        88. A method of detecting a disease characterized by protein misfolding, the method comprising:

- (a) providing a test protein suspected of being misfolded;
- 20        (b) contacting the protein with an exchange buffer comprising a denaturant and deuterium, the exchange buffer having a denaturant concentration;
- (c) contacting the test protein with a mass spectrometry matrix medium;
- 25        (d) determining a change in mass of the test protein by mass spectrometry;
- (e) varying the denaturant concentration of the exchange buffer;
- (f) repeating steps (a)-(e) a desired number of times; and
- (g) analyzing the change in mass of the test protein to determine a stability of the test protein; and
- 30        (h) comparing the stability of the test protein suspected of being misfolded with a known stability of the test protein, wherein a change in stability of the test protein when compared with the

known stability of the test protein is indicative of a disease characterized by protein misfolding.

89. The method of claim 88, wherein the test protein is disposed in  
5 a crude cell lysate.

90. The method of claim 88, wherein the test protein has a mass of less than 1,000,000 daltons.

10 91. The method of claim 88, wherein the test protein is a multimeric protein.

92. The method of claim 88, wherein the test protein is disposed on a microtiter plate.  
15

93. The method of claim 92, wherein a plurality of test proteins are disposed on the microtiter plate.

94. The method of claim 93, wherein the method further comprises  
20 the step of repeating steps (a)-(h) for each test protein disposed on the microtiter plate.

95. The method of claim 88, wherein the test protein is provided in picomolar or greater amounts.  
25

96. The method of claim 88, wherein the test protein is *in vivo*.

97. The method of claim 88, wherein the denaturant is a chemical denaturant.  
30

98. The method of claim 97, wherein the denaturant is selected from the group consisting of detergents, guanidinium chloride and urea.

99. The method of claim 88, wherein the mass spectrometry matrix material is a MALDI mass spectrometry matrix material and the mass spectrometry is MALDI mass spectrometry.

100. The method of claim 99, wherein the MALDI mass spectrometry matrix material is selected from the group consisting of sinapinic acid,  $\alpha$ -cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, 2,5-dihydroxyacetophenone and 3-amino-4-hydroxybenzoic acid.

101. The method of claim 88, wherein the analyzing comprises fitting data comprising a change in mass of the mutant test protein as a function of denaturant to the equation  $\Delta\text{Mass} = \Delta M_{\infty} + (\Delta M_0 - \Delta M_{\infty})e^{-(k_{\text{int}}/(1+K_{\text{fold}}))t}$ , wherein  $K_{\text{fold}} = e^{-(\Delta G_f + m[\text{Denat.}]/RT)}$  and  $\Delta G_f$  is the stability of the mutant test protein.

102. The method of claim 88, wherein the analyzing is performed using a computer program.

103. The method of claim 88, wherein the step of analyzing the change in mass of the test protein to determine a stability of the mutant test protein comprises determining a free energy of folding from a denaturation curve based on the change of mass of the protein, the free energy of folding being indicative of the stability of a test protein.

104. The method of claim 88, wherein the step of analyzing the change in mass of the test protein to determine a stability of the mutant test protein comprises determining a change in mass of the test protein based on the exchange of deuterium for hydrogen, the exchange being dependent on slow-exchanging hydrogens in the test protein.

105. The method of claim 88, further comprising providing a reference protein with the test protein.

106. The method of claim 88, further comprising:

- 5
- (a) contacting the test protein with a reverse phase chromatography matrix; and
  - (b) eluting the test protein from the chromatography matrix.

107. The method of claim 106, further comprising contacting the test protein with an acid before contacting the test protein with the reverse phase chromatography matrix.

10

108. A method of identifying a protein that unfolds through one or more stable intermediates, the method comprising:

- 15
- (a) providing a test protein suspected of unfolding through one or more intermediates;
  - (b) contacting the test protein with an exchange buffer comprising a denaturant and deuterium, the exchange buffer having a denaturant concentration;
  - 20 (c) contacting the test protein with a mass spectrometry matrix medium;
  - (d) determining a change in mass of the test protein by mass spectrometry;
  - (e) varying the denaturant concentration of the exchange buffer;
  - 25 (f) repeating steps (a)-(e) a desired number of times; and
  - (g) plotting the change in mass of the test protein as a function of denaturant concentration to generate an unfolding curve;
  - (h) evaluating the unfolding curve, to thereby identify one or more stable intermediates.
- 30

109. The method of claim 108, wherein the test protein is disposed in a crude cell lysate.

110. The method of claim 108, wherein the test protein has a mass of less than 1,000,000 daltons.

5        111. The method of claim 108, wherein the test protein is a multimeric protein.

112. The method of claim 108, wherein the test protein is disposed on a microtiter plate.

10        113. The method of claim 108, wherein a plurality of test proteins are disposed on the microtiter plate.

114. The method of claim 113, wherein the method further comprises the step of repeating steps (a)-(h) for each test protein disposed on the microtiter plate.

115. The method of claim 108, wherein the test protein is provided in picomolar or greater amounts.

20        116. The method of claim 108, wherein the test protein is *in vivo*.

117. The method of claim 108, wherein the denaturant is a chemical denaturant.

25        118. The method of claim 117, wherein the denaturant is selected from the group consisting of detergents, guanidinium chloride and urea.

119. The method of claim 108, wherein the mass spectrometry matrix material is a MALDI mass spectrometry matrix material and the mass spectrometry is MALDI mass spectrometry.

30

120. The method of claim 119, wherein the MALDI mass spectrometry matrix material is selected from the group consisting of sinapinic acid,  $\alpha$ -cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, 2,5-dihydroxyacetophenone and 3-amino-4-hydroxybenzoic acid.

5

121. The method of claim 108, wherein the plotting is performed using a computer program.

122. The method of claim 108, further comprising providing a  
10 reference protein with the test protein.

123. The method of claim 108, further comprising:

- (a) contacting the test protein with a reverse phase chromatography matrix; and
- 15 (b) eluting the test protein from the chromatography matrix.

124. The method of claim 123, further comprising contacting the test protein with an acid before contacting the test protein with the reverse phase chromatography matrix.

20